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(54) Title: CHITOSAN INDUCED IMMUNOPOTENTIATION

(57) Abstract: Methods and compositions for potentiating an immune response are disclosed which incorporate chitosan as an immunopotentiating adjuvant. Administration of the compositions of the invention is effected by various routes.

WO 01/35994 A2

CHITOSAN INDUCED IMMUNOPOTENTIATION

This is a continuation-in-part of U.S. application no. 08/823,143, filed March 25, 1997.

FIELD OF THE INVENTION

The present invention relates generally to methods for potentiating an immune response in an animal, compositions to effect the potentiation, and methods to produce the compositions. More specifically, the invention provides methods comprising the use of an antigen/chitosan mixture or an antigen/chitosan/oil/surfactant emulsion to potentiate an immune response, antigen/chitosan mixtures or antigen/chitosan/oil/surfactant emulsions to effect potentiation, and methods to prepare the antigen/chitosan mixture or antigen/chitosan/oil emulsion.

BACKGROUND OF THE INVENTION

Recent biotechnological advances have facilitated identification and isolation of components in complex antigens which provide prospects for successful development of safe and practical vaccines. Often, however, these isolated components are not as immunogenic as the complete complex antigens from which they were derived. In order to enhance an immune response to a weakly antigenic immunogen in a recipient animal, adjuvants are frequently administered with the immunogen. Despite the universal acceptance of adjuvants, however, the number suitable for use in humans is limited.

Ideally, an adjuvant should potentiate long-lasting expression of functionally active antibodies, elicit cell-mediated immunity (CMI), and enhance production of memory T- and B-lymphocytes with highly specific immunoreactivity against an invading antigen. In addition to providing a defense upon immediate challenge with an foreign antigen, these responses should provide protection against any future encounters of the host with a specific antigen. More important is the ability of an adjuvant to augment the immune response with a minimum of toxic side effects. Therefore, efficacy of an adjuvant is described in terms of how it balances positive

(potentiated immunity) and negative (toxicity) influences.

Controlled immunization for the purpose of stimulating antibody production by B cells is dependent upon a myriad of factors inherent to both the antigen itself and the immunized animal. In general, the farther removed in evolutionary terms the antigen, or its source, is from the invaded host, the more effective the immune response elicited by the antigen. Antigens derived from closely related species are less competent in eliciting antibody production due to the fact that the host immune system is sometimes unable to clearly distinguish the foreign antigen from endogenous, or self antigens. In addition, the dosage of the antigen, the purity of the antigen, and the frequency with which the antigen is administered are also factors which significantly contribute to the resulting antibody titer and specificity of the resulting antibodies. Still other factors include the form, or complexity, of the antigen, and how the antigen is administered. Finally, both the genetic makeup and overall physiological state of the immunized animal contribute to the extent to which an immune response is mounted. Of these factors, the form or complexity of the antigen is directly affected by immunization with an adjuvant.

Current understanding suggests that adjuvants act to augment the immune response by a variety of different mechanisms. In one mechanism, the adjuvant directly stimulates one of either $CD4^+$ helper T-cell subpopulations designated T_H1 or T_H2 [Mosmann and Coffman, *Ann. Rev. Immunol.* 7:145-173 (1989)]. Helper T cells are required for B-cell antibody responses to most antigens. In an appropriate immune response, an antigen is captured and processed by an antigen-presenting cell (APC), *e.g.*, circulating or tissue macrophages, and presented on the surface of the APC in association with a class II major histocompatibility (MHC) molecule. In this form, the antigen can interact with receptors on the surface of helper T cells thereby activating the particular subpopulation of cells to express and secrete any of a number of cytokines. The nature of cytokine production depends on the subset of helper T cells activated, a result that can be modulated in part by the choice of adjuvant. For example, alum, an aluminum salt adjuvant approved for clinical use in humans, has been reported to selectively activate T_H2 cells in mice [Grun and Maurer, *Cell. Immunol. 121*:134-145 (1989)], while Freund's complete adjuvant (CFA), an emulsion of mineral oil with killed mycobacteria [Freund,

et al., Proc. Soc. Exp. Biol. Med. 37:509 (1937)], preferentially activates murine T_H1 cells [Grun and Maurer, *Cell. Immunol. 121*:134-145 (1989)].

Another mechanism by which the immune response is augmented involves the direct stimulation of B cells by, for example, lipopolysaccharide (LPS) from Gram-negative bacteria. [Gery, *et al., J. Immunol. 108*:1088 (1972)]. LPS has also been shown to stimulate secretion of interferon-γ (INF-γ) [Tomai and Johnson, *J. Biol. Resp. Med. 8*:625-643 (1989)], which both inhibits proliferation of T_H2 cells and stimulates differentiation of T_H1 cells [Gajewski, *et al., J. Immunol. 143*:15-22 (1989); Gajewski, *et al., J. Immunol. 146*:1750-1758 (1991)]. The mechanism by which LPS potentiates the immune response is therefore through direct stimulation of B cells, and indirect regulation of both T_H1 and T_H2 cell populations.

Still other modes of immunopotentiation have been reported for other adjuvants. Oil emulsions (*i.e.*, Complete Freund's Adjuvant [CFA], Freund's incomplete adjuvant [FIA]) and liposomes act through depot formation as does alum, thus allowing for slow release of antigen. Slow release of antigen permits extended exposure of the antigen to the immune system and also allows for initial immunization with a dosage of antigen that, if delivered at one time, would ordinarily be counterproductive to antibody formation. It has been previously reported that while a large initial dose of antigen results in the production of a higher immediate titer of antibody, the increase in antibody titer and increase in antibody specificity as a function of time is not as great as observed with lower and more frequent doses of antigen [Siskind, G., *Pharm. Rev. 25*:319-324 (1973)]. Therefore, adjuvants which control presentation of an antigen to the immune system modulate antigen dosage in addition to altering the form, or complexity, of the antigen.

To date, only one adjuvant, alum [$AlK(SO_4)_2 \cdot H_2O$], has proven sufficiently non-toxic to permit its use in humans. Alum not only acts through T_H2 cell activation, depot formation and slow release of antigen following immunization [Edelman, *Rev. Infect. Dis. 2*:370-383 (1980); Warren, *et al., Ann. Rev. Immunol. 4*:369-388 (1986)], but also through granuloma formation by attracting immunocompetent cells [White, *et al., J. Exp. Med. 102*:73-82 (1955)] and activation of complement [Ramanathan, *et al., Immunol. 37*:881-888 (1979)]. However, alum is not without its negative side effects which include erythema, subcutaneous nodules, contact hypersensitivity, and

granulomatous inflammation. Other adjuvants, which are widely employed outside of human application, are also the focus of continuing research to develop acceptable alternatives for use in humans. Included are the above mentioned oil emulsions (*i.e.*, CFA and FIA), bacterial products (*i.e.*, LPS, cholera toxin, mycobacterial components and whole killed *Corynebacterium parvum*, *Corynebacterium granulosum*, and *Bordetella pertussis*, liposomes, immunostimulating complexes (ISCOMs), and naturally occurring and derivatized polysaccharides from other than bacterial sources.

The immunopotentiating capacity of polysaccharides has been a focus of investigation over the past few years as these compounds are widespread in nature, *e.g.*, as structural components in the cell walls of bacteria, and exoskeletons of insects and crustacea. Lipopolysaccharide (LPS) isolated from certain Gram-negative bacteria is one such polysaccharide even though the adjuvant properties of LPS are derived mainly from the lipid A region of the molecule, and not from the *o*-specific polysaccharide or core oligosaccharide regions of the molecule. LPS, which augments both humoral [Johnson, *et al., J. Exp. Med. 103*:225-246 (1956)] and cell-mediated immunity [Ohta, *et al., Immunobiology 53*:827 (1984)], possesses numerous biological activities, but is impractical for use in humans due to its inherent toxicity as reviewed by Gupta, *et al., Vaccine 11*:291-306 (1993). Attention has therefore shifted to other polysaccharides including, among others, chitosan.

Chitosan [β-(1-4)-2-amino-2-deoxy-D-glucan] is a derivative of chitin and has been widely used in biomedical applications, due in part to is biodegradability by lysozyme and low toxicity in humans. These same properties have resulted in increased interest in chitosan as an immunopotentiating agent. For example, Matuhashi, *et al.*, in U.S. Patent No. 4,372,883, disclosed conjugation of soluble polysaccharides, including chitosan, to normally toxic antigens, conjugation thereby detoxifying the antigen and permitting its use as an immunogen. Matuhashi *et al.*, however, did not address the use of insoluble forms of chitosan, nor did Matuhashi compare the resulting serum antibody titer with that obtained from immunization with other known adjuvants.

Likewise, Suzuki, *et al.*, in U.S. Patent 4,971,956, disclosed the use of water soluble chitosan-oligomers as therapeutics for treatment of bacterial and fungal infections, as well as for the treatment of tumors. Suzuki, *et al.* discussed the difficulty

in modifying chitosan to produce an appropriate water soluble form, disclosing that water-insoluble forms are impractical for therapeutic application. In addition, Suzuki *et al.*, does not disclose conjugation of an antigen to chitosan to effect enhanced immune response.

Mitsuhashi, *et al.*, in U.S. Patent 4,814,169, disclosed the use of human protein conjugated to soluble polysaccharides, including chitosan, to generate antibodies against human protein in non-human animals. Administration of the human protein/polysaccharide solution was by intravenous, intraperitoneal, or subcutaneous injection. Other routes, including oral and rectal administration, were not addressed in the disclosure.

Nishimura, *et al.* [*Vaccine 2*:93-99 (1984)] reported the immunological properties of derivatives of chitin in terms of activation of peritoneal macrophages *in vivo*, suppression of tumor growth in mice, and protection against bacterial infection. Results suggested that both chitin and chitosan were ineffective stimulators of host resistance against challenge with tumor cells or bacteria, but that chitosan moderately induced cytotoxic macrophages. Results with modified, de-acetylated chitosan, which forms a gel in an aqueous environment, was shown to more effectively activate macrophages, suppress tumor growth and stimulate resistance to bacterial infection.

Marcinkiewicz, *et al.*, [*Arch. Immunol. Ther. Exp. 39*:127-132 (1991)] examined the immunoadjuvant activity of water-insoluble chitosan and reported significant enhancement of T-dependent humoral response, but only moderate augmentation of T-independent humoral response. The enhanced humoral response was detected with chitosan at doses of 100 mg/kg administered either intravenously or intraperitoneally. Subcutaneous and oral administration were specifically reported as being ineffective. In addition, Marcinkiewicz, *et al.*, does not suggest conjugation of an antigen to insoluble chitosan, stating that chitosan "resulted in the same response irrespective of the site of administration - either together or separately from antigen."

In light of the fact that only one existing adjuvant has been approved for use in humans, there thus exists a need in the art to provide novel and less toxic adjuvants for potential application in humans. Improved adjuvants will permit the production of more effective vaccines and will improve the production of monoclonal antibodies with

therapeutic potential.

SUMMARY OF THE INVENTION

In all of its aspects, the invention is directed to the use of chitosan formulations for potentiating an immune response in a host.

In one aspect, the present invention is directed to a method for potentiating an immune response comprising the steps of preparing a chitosan solution, incorporating an antigen into a phosphate buffer to form an antigen/phosphate buffer solution, lyophilizing the antigen/phosphate buffer solution to a lyophilized mixture, reconstituting the lyophilized mixture with the chitosan solution to form an antigen/chitosan mixture, and administering the mixture to an animal, including humans. The antigen/chitosan mixture may be administered to the animal via oral, rectal, intravaginal routes as well as via intraperitoneal injection, intramuscular injection, or subcutaneous injection; administration may comprise a single route or a multiplicity of routes.

In another aspect of the invention, a composition is provided which, comprises in combination lyophilized antigen/phosphate buffer and chitosan solution. The antigen/chitosan mixture may be administered to the animal via oral, rectal, intravaginal routes as well as via intraperitoneal injection, intramuscular injection, or subcutaneous injection; administration may comprise a single route or a multiplicity of routes.

Also provided by the invention is an immunogen comprising a lyophilized antigen/phosphate buffer and chitosan solution. The antigen/chitosan mixture may be administered to the animal via oral, rectal, intravaginal routes as well as via intraperitoneal injection, intramuscular injection, or subcutaneous injection; administration may comprise a single route or a multiplicity of routes.

In another aspect of the invention, a method is provided for preparing an immunogen comprising, preparing a chitosan solution, incorporating an antigen into a phosphate buffer to form an antigen/phosphate buffer solution, lyophilizing the antigen/phosphate buffer solution to a lyophilized mixture, and reconstituting the lyophilized mixture with the chitosan solution to form an antigen/chitosan mixture.

As another aspect of the invention, the present invention provides a

method for potentiating an immune response comprising the steps of preparing a chitosan solution, preparing a sodium hydroxide solution, preparing an oil/surfactant solution, wherein the oil can be metabolically degraded, mixing the chitosan solution with the sodium hydroxide solution, the oil/surfactant solution, and the antigen to form an emulsion, and administering the emulsion to an animal. The antigen may be, but is not limited to, a protein, carbohydrate, lipid, glycoprotein or combinations thereof. Preferably the pH of the chitosan solution is about 5.0. The emulsion may be administered to the animal via intraperitoneal injection, intramuscular injection, or subcutaneous injection. The emulsion may also be administered alone, or in combination with any of a number of other adjuvants. Immunization may comprise a single administration or a multiplicity of administrations. In a more preferred embodiment, the oil is squalene.

In yet another aspect of the invention, a composition is provided which, when administered to an animal, will potentiate an immune response, the composition comprising antigen, sodium hydroxide, oil, surfactant, and chitosan solution, wherein the oil can be metabolically degraded.

Also provided by the invention is an immunogen comprising an antigen, sodium hydroxide solution, oil, surfactant, and a chitosan solution, wherein the oil can be metabolically degraded.

In another aspect of the invention, a method is provided for preparing an immunogen comprising, of preparing a chitosan solution, preparing a sodium hydroxide solution, preparing an oil/surfactant solution, wherein the oil can be metabolically degraded, mixing the chitosan solution with the sodium hydroxide solution, the oil/surfactant solution, and the antigen to form an emulsion.

Also provided in another aspect of the invention is a kit comprising a chitosan solution, a sodium hydroxide solution, and an oil/surfactant solution.

As another aspect of the invention, the present invention provides a method for potentiating an immune response comprising the steps of preparing a chitosan solution, preparing a phosphate buffer solution, preparing an oil/surfactant solution, wherein the oil can be metabolically degraded, mixing the chitosan solution with the phosphate buffer and/or HEPES buffer, the oil/surfactant solution, and the antigen to

form an emulsion, and administering the emulsion to an animal. The antigen may be, but is not limited to, a protein, carbohydrate, lipid, glycoprotein or combinations thereof. Preferably the pH of the chitosan solution is about 5.0. The emulsion may be administered to the animal via intraperitoneal injection, intramuscular injection, or subcutaneous injection. The emulsion may also be administered alone, or in combination with any of a number of other adjuvants. Immunization may comprise a single administration or a multiplicity of administrations. In a more preferred embodiment, the oil is squalene.

In yet another aspect of the invention, a composition is provided which, when administered to an animal, will potentiate an immune response, the composition comprising antigen, phosphate buffer and/or HEPES buffer, oil, surfactant, and chitosan solution, wherein the oil can be metabolically degraded.

Also provided by the invention is an immunogen comprising an antigen, phosphate buffer solution, oil, surfactant, and a chitosan solution, wherein the oil can be metabolically degraded.

In another aspect of the invention, a method is provided for preparing an immunogen comprising, of preparing a chitosan solution, preparing a phosphate buffer and/or a HEPES buffer, preparing an oil/surfactant solution, wherein the oil can be metabolically degraded, mixing the chitosan solution with the phosphate buffer and/or HEPES buffer, the oil/surfactant solution, and the antigen to form an emulsion.

Also provided in another aspect of the invention is a kit comprising a chitosan solution, a phosphate buffer and/or a HEPES buffer, and an oil/surfactant solution.

DETAILED DESCRIPTION OF THE INVENTION

The present invention is illustrated by the following examples relating to compositions and methods for using compositions for immunopotentiation which comprise an antigen/chitosan mixture or an antigen/chitosan/oil/surfactant emulsion, as well as methods to prepare the antigen/chitosan mixture and the

antigen/chitosan/oil/surfactant emulsion. In particular Example 1 demonstrates the preparation of antigen incorporated and lyophilized in phosphate buffer, which is subsequently reconstituted in a chitosan solution. Example 2 provides a comparison of the ability of antigen incorporated into phosphate buffer and reconstituted in a chitosan solution to stimulate an immune response to that of a currently available adjuvant. Example 3 demonstrates the preparation of antigen incorporated in a chitosan/oil emulsion. Examples 4 and 5 provide a comparison of the ability of different antigens incorporated into a chitosan/oil emulsion to stimulate an immune response to that of a currently available adjuvant. Example 6 demonstrates the preparation of antigen incorporated into an alternative chitosan/oil emulsion, while Example 7 provides a comparison of the ability of alternative chitosan/oil emulsions to stimulate an immune response.

Example 1

Preparation of Antigen Incorporated and Lyophilized in Phosphate Buffer and Reconstituted in Chitosan Solution

While the following is exemplified by the use of chicken ovalbumin as an antigen, those of ordinary skill in the art will readily appreciate that any number of other antigens may be employed.

A 0.5 M phosphate buffer was prepared by diluting 15.6 ml of phosphoric acid (16 M; Mallinkrodt Chemical, Paris, KY) in 400 ml of deionized (18 mOhm: DI) water. The pH of the solution was adjusted to 7.3 with 10 N sodium hydroxide (Sigma Chemical Co., St. Louis, MO). The total volume of the solution was adjusted to 500 ml by the addition of DI water.

A dilute chitosan solution was made by first preparing a 1% chitosan in 2% acetic acid solution: 1 gm of chitosan (practical grade; Sigma Chemical Co., St. Louis, MO) in 100 ml of 2% glacial acetic acid (Mallinkrodt Chemical, Paris, KY). The resulting 1% chitosan in 2% acetic acid solution was then diluted further by adding 7.4 ml of the solution to 2.6 ml of DI water to obtain a chitosan working solution. The pH of the final chitosan solution was between 6 and 7.

50 μL of a 10mg/ml ovalbumin (Sigma Chemical Co., St; Louis, MO) solution in phosphate-buffered saline was added to a 10 ml vial containing 5 ml of the 0.5 M phosphate buffer. This resulted in a clear flocculent. After adding 0.5 gm of *d*-sorbitol (Sigma Chemical Co., St. Louis, MO), the solution was rapidly frozen in liquid nitrogen and lyophilized.

Lyophilized sample was reconstituted with 5 ml of the working chitosan solution, mixed by vortex to form a cloudy solution containing white particles, and used for immunization as described in Example 2.

Example 2

Comparative Immunopotentiation with Antigen Incorporated and Lyophilized in Phosphate Buffer and Reconstituted in Chitosan Solution

In order to determine the relative degree to which chisotsan potentiated the response to an antigen, a comparison (see Table 1) was undertaken between groups of mice that were previously immunized (individually) with either a vaccine comprising 25 μg of ovalbumin with CFA (Sigma Chemical Co., St. Louis, MO) or a vaccine comprising 25 μg of ovalbumin incorporated and lyophilized in phosphate buffer, and subsequently reconstituted in a chitosan solution (Test Group), as prepared in Example 1.

Female Balb/c mice, 8 weeks of age, were immunized by a single intraperitoneal injection of the vaccine on day 0. The ovalbumin CFA treament group contained 3 mice, while the test group (treated ovalbumin incorporated and lyophilized in phosphate buffer and reconstituted in a chitosan solution) contained 4 mice. Both experimental groups were bled on day 7, post-injection. The CFA adjuvanted group was also bled on days 21, 28, 35, 42, and 48 post-immunization. The test group was also bled on days 26, 38, 38, 52, 70, 83, 102, 123, and 159 post-immunization. Anti-ovalbumin serum antibody titers were determined by ELISA.

Table 1

Comparative Immunopotentation with Antigen Incorporated and Lyophilized in Phosphate Buffer and Reconstituted in a Chitosan Solution

(compilation of experiments)

Day Post-Immunization	TEST GROUP (mean antibody titer)	CFA (mean antibody titer)
7	1:100	1:73
21		1:293
26	1:10,000	
28		1:3,200
35		1:13,867
38	1:11,000	
42	booster immunization administered (Test Group only)	1:15,360
48		1:20,480
52	1:64,000	
70	1:18,500	
83	1:10,250	
102	1:13,500	
123	1:10,000	
159	1:1750	

The results indicated that the composition comprising antigen incorporated and lyophilized in phosphate buffer and reconstituted in chitosan solution was apparently non-toxic to the recipient animals. The test group animals developed a high antibody titer by day 26 (10,000). The high titer persisted past 83 days post-immunization, via a booster vaccination on day 42. Immediately following the booster

vaccination, the titer increased to appproximately 64,000 (day 52) and persisted above 10,000 to approximately 123 days post-vaccination (original). The Test Group values obtained were comparable to those of the standard adjuvant used by those of ordinary skill in the art, Complete Freund's Adjuvant. Further, the mean titer values in the test group animals were comparable to those seen with antigens cross-linked to chitosan with glutaraldehyde, which generally improves immunopotentiation over other commercially available adjuvants (PCT/US95/12189; WO 96/09805). In view of the unacceptability of glutaraldehyde in commercial vaccines and the present vaccine, wherein the antigen is administered in via incorporation and lyophilization in phosphate buffer and recconstituton in a chitosan solution, the present invention is a safe and comparable alternative adjuvant to both CFA and antigens cross-linked to chitosan via glutaraldehyde.

Example 3

Preparation of an Antigen Incorporated into a Chitosan / Oil Emulsion

While the following is exemplified by the use of HIV-peptide-keyhole limpet hemocyanin conjugate (Example 4) or human zona pellucida B peptide-ovalbumin (Example 5) as antigens, those of ordinary skill in the art will readily appreciate that any number of other antigens may be employed. Further while the following is exemplified by the use of squalene, those of ordinary skill in the art will appreciate that any oil that is readily metabolized by the recipient animal may be used (*e.g.*, corn, canola, peanut).

A 2% chitosan solution in 0.5 M sodium acetate was prepared by dissolving 4.1 g of sodium acetate (Sigma Chemical Co., St. Louis, MO) in 50 ml of deionized (18 mOhm: DI) water with mixing. The pH of the solution was adjusted to 4.5 with approximately 7 ml of glacial acetic acid (Mallinkrodt Chemical, Paris, KY) and an additional 1.5 ml of glacial acetic acid was added to compensate for the effect of the addition of chitosan on the pH of the solution. The total volume of the solution was adjusted to 100 ml by the addition of DI water. 2 grams of chitosan (Sigma Chemical Co., St. Louis, MO) was slowly added to the sodium acetate solution with stirring and the mixture was stirred for 2-3 hours until the chitosan had dissolved. The chitosan solution

was then sterilized by autoclaving during a 25 minute cycle. The solution was cooled to room temperature in a biosafety cabinet. The chitosan solution was then clarified by centrifugation in an IEC clinical centrifuge (International Equipment Co., Needham Hts., MA) at setting 7 for 5 minutes. The supernatant was decanted from the pellet (insoluble chitosan/chitin and contaminants). 87 to 90% (by weight) of the chitosan added was retained in the supernatant.

A 50% sodium hydroxide solution was prepared by dissolving 50 gm of sodium hydroxide (Sigma Chemical Co., St. Louis, MO) in 100 ml of deionized water, with mixing. A squalene/surfactant solution was prepared by combining 1500 μL of squalene (2,6,10,15,19,23-Hexamethyl-2,6,10,14,18,22-tetracosahexaene; Sigma Chemical Co., St. Louis, MO) with 600 μL of the surfactant Pluronic® L121 (BASF Corp., Parsippany, NJ) and vortexed until homogeneous.

A chitosan/squalene/surfactant/antigen emulsion was prepared by adding approximately 420 μL of antigen (*i.e.*, HIV-peptide-keyhole limpet hemocyanin conjugate, Table 2; human zona pellucida B peptide-ovalbumin conjugates, Table 3) in water or urea to approximately 370 μL of 2% chitosan in 0.5 M sodium acetate and vortexing. The actual amount of antigen (*i.e.*, protein or peptide-carrier conjugate) used may range from 1 μg to several milligrams. 10 μL of the 50% sodium hydroxide were then added to the antigen/chitosan and the sample was vortexed. 10 μL aliquots of the 50% sodium hydroxide were added until a stable cloudy precipitate formed. Approximately 140 μL of the previously prepared squalene/surfactant solution was added to the above solutions of antigen & chitosan. The resulting solution was vortexed until a cloudy emulsion formed. Immediately prior to administration in the immunization studies as described in Examples 4 and 5, the resulting solution of chitosan/squalene/surfactant/antigen was mixed by vortexing or syringe aspiration.

Example 4

Comparative Immunopotentiation with

Antigen (HIV-peptide-KLH-conjugate)

Incorporated into a Chitosan/Squalene Emulsion

The following experiments were conducted in order to assess the immune response to an antigen that has been incorporated into a chitosan/squalene emulsion. Specifically a comparative study was undertaken wherein groups of mice were individually immunized with either a vaccine comprising various amounts of HIV-peptide-KLH conjugate [Saren *et al. Vaccine Res.*, 3:49-57; incorporated herein by reference] with the chitosan/squalene/surfactant emulsion or 20 μg of HIV-peptide-KLH conjugate with CFA.

Referring to Tables 2 and 3, female, Balb/c mice, 8 weeks of age were immunized by a single 200 μL intraperitoneal injection of the vaccine on day 0. A second immunization was given to Group 1, at week 18 (126 days after the first immunization). A second immunization was adminsitered to Groups 2 and 3 at week 24 (168 days after the first immunization). The second immunization consisted of the unconjugated HIV peptide at the dosage indicated with the chitosan/squalene/surfactant emulsion in Groups 1-3. The CFA group did not receive a second immunization. The subject animals were bled on days 22, 35, 49, 63, 77, 91, 119 (excluding Group 1), 140, and 149. Serum antibody titers were determined by ELISA.

Table 2

Immunization Groups in

Comparative Immunopotentiation Studies with

Chitosan/Squalene Emulsion

Group #	μg of peptide	Adjuvant	No. of animals
1	1	chitosan/squalene	8
2	3	chitosan/squalene	4
3	20	chitosan/squalene	4
4	20	CFA	4

Table 3

Comparative Immunopotentiation with

Chitosan / Squalene Emulsion

Geometric mean antibody titer at week:

Grp #	3	5	7	9	11	13	17	20	27
1	1:115	1:475	1:2260	nd	1:4000	1:2500	nd	1:35910	nd
2	1:126	1:6400	1:9190	1:11310	1:9120	1:1130	1:2000	1:707	1:18000
3	1:141	1:4500	1:18400	1:9500	1:12900	1:1900	1:3360	1:1680	1:9120
4	1:62	1:2600	1:3820	1:800	1:9120	1:3200	1:4700	1:2200	nd

nd = not determined

The results indicated that the chitosan/squalene/surfactant emulsion adjuvant was apparently non-toxic to the recipient animals. The results also show that the Group 3 (20 μg peptide adjuvanted with chitosan/squalene/surfactant) performed as well as Group 4 (20 μg peptide adjuvanted with CFA), with an improved immune response in weeks 5 and 7 against the HIV-peptide-KLH conjugate. Further, Group 3 (3 μg peptide adjuvanted with chitosan/squalene/surfactant) produced results that were similar, if not better than Group 4 through week 11. Surprisingly, a second immunization with *unconjugated* peptide in groups receiving the chitosan/squalene emulsion resulted in a very strong boost response (see Group 1, post-week 18 and Groups 2-3, post-week 24). Overall, the results set forth in Table 3 demonstrate that the chitosan/squalene emulsion induces a comparable, and in some cases better, humoral immune response than does CFA. Additionally, the chitosan/squalene/surfactant emulsion acted as an immunopotentiator as shown by a very strong boost response obtained with unconjugated HIV peptide.

Example 4

Comparative Immunopotentiation with

Antigen (human zona pellucida B peptide-ovalbumin conjugates)

Incorporated into a Chitosan / Squalene Emulsion

The following experiments were also conducted to assess the immune response to an antigen that has been incorporated into a chitosan/squalene/surfactant emulsion. Specifically a comparative study was undertaken wherein groups of mice were individually immunized (intraperitoneal) with a vaccine comprising 6 different human zona pellucida B (ZPB) synthetic peptides [SEQ ID NOS. 1-6] adjuvanted with either the chitosan/ squalene/surfactant emulsion or CFA.

Female Balb/c mice, 8 weeks of female, were immunized by a 200 μL intraperitoneal injection of the vaccine (20 μg each of 6 different human ZPB synthetic peptides combined either with chitosan/squalene/surfactant emulsion (Group I) or CFA (Group II) on days 0 and 28. The Group II mice received CFA vaccine as the booster. Serum antibody titers were determined by ELISA using plates

coated with 1 μg per well of a mixture of the 6 peptides. Antibody titers against full length purified recombinant human ZPB protein produced in Chinese hamster ovary cells [Harris *et al. J. Seq. and Mapping*, 4:361-393, 1994; incorporated herein by reference] were also determined by ELISA on plates coated with 50 ng of purified protein.

Table 4

Comparative Immunopotentiation with

Chitosan / Squalene Emulsion

(data expressed as geometric mean)

	Peptide specific antibody titers at day:				Anti-CHO ZPB antibody titers at day:		
Adjuvant	21	43	60	81	43	60	1:81
Group I	1:4.47	1:30900	1:43700	1:87450	1:1350	1:2390	1:9120
Group II	1:9.45	1:8360	1:4550	1:11870	1:146	1:2.8	1:10

The results in Table 4 demonstrate that the animals immunized with the peptide-conjugate with chitosan/squalene/surfactant emulsion elicited a humoral response to both peptide and full-length protein superior to that elicited by immunization with peptide-conjugate adjuvanted with CFA.

Example 5

Preparation of an Antigen Incorporated into an Alternative Chitosan / Oil Emulsion

While the following is exemplified by the use of squalene, those of ordinary skill in the art will appreciate that any oil that is readily metabolized by the recipient animal may be used (*e.g.*, corn, canola, peanut).

As an alternative to the chitosan/oil emulsion disclosed in Example 3, the chitosan/oil emulsion of the present example is preferable for use with antigens that are sensitive to epitope (which are important for the induction of immunity) degradation via interaction with strong bases or in situations where titration with the base is critical (*i.e.*, where additional buffering capacity may be desirable).

A 2% chitosan solution in 0.5 M sodium acetate was prepared by dissolving 4.1 g of sodium acetate (Sigma Chemical Co., St. Louis, MO) in 50 ml of deionized (18 mOhm: DI) water with mixing. The pH of the solution was adjusted to 4.5 with approximately 7 ml of glacial acetic acid (Mallinkrodt Chemical, Paris, KY) and an additional 1.5 ml of glacial acetic acid was added to compensate for the effect of the addition of chitosan on the pH of the solution. The total volume of the solution was adjusted to 100 ml by the addition of DI water. 2 grams of chitosan (Sigma Chemical Co., St. Louis, MO) was slowly added to the sodium acetate solution with stirring and the mixture was stirred for 2-3 hours until the chitosan had dissolved. The chitosan solution was then sterilized by autoclaving during a 25 minute cycle. The solution was cooled to room temperature in a biosafety cabinet. The chitosan solution was then clarified by centrifugation in an IEC clinical centrifuge (International Equipment Co., Needham Hts., MA) at setting 7 for 5 minutes. The supernatant was decanted from the pellet (insoluble chitosan/chitin and contaminants). 87 to 90% (by weight) of the chitosan added was retained in the

supernatant. At this point the chitosan solution may be dialyzed overnight to reduce ion concentration.

Alternatively, a 2% chitosan solution may be prepared by dissolving chitosan salts (Pronova Biomedical, Oslo, Norway) in water and sterilizing by autoclaving. As a further alternative, a 2% chitosan solution may be prepared via preparation of chitosan salts by the following method. Specifically, chitosan (Sigma Chemical, St. Louis, MO or CTC Organics, Atlanta, GA) is dissolved in a 1% hydrochloric acid solution. Concentrated hydrochloric acid is added until precipitation of the crude chitosan-HCL is complete. The precipitated chitosan salt is than washed with ethanol and dried. The resulting chitosan salt is dissolved in water to a 2% solution, dialyzed extensively against water and sterilized by autoclaving.

A squalene/surfactant solution was prepared by combining 1500 μL of squalene (2,6,10,15,19,23-Hexamethyl-2,6,10,14,18,22-tetracosahexaene; Sigma Chemical Co., St. Louis, MO) with 600 μL of the surfactant Pluronic® L121 (BASF Corp., Parsippany, NJ) and vortexed until homogeneous.

A chitosan/squalene/surfactant/antigen emulsion was prepared by adding approximately 450 μL of antigen in phosphate-buffered saline to approximately 350 μL of a sterile dialyzed 2% chitosan solution and vortexing. The actual amount of antigen (*i.e.*, protein or peptide-carrier conjugate) used may range from 1 μg to several milligrams. In order to neutralize/precipitate the chitosan/antigen complex, 250 μL of the 0.2 M phosphate buffer (although a phosphate buffer is exemplified, the use of a HEPES [(N-[2-Hydroxyethyl]piperazine-N`-[2ethanesulfonic acid])] buffer or similar biological buffers having a pH buffering range of 6.5 to 8.0 are also contemplated) were then added to the antigen/chitosan and the sample was vortexed. Approximately 150 μL of the previously prepared squalene/surfactant solution was added to the above solutions of antigen & chitosan. The resulting solution was vortexed until a cloudy emulsion formed. Immediately prior to administration in the immunization studies as described in Example 6, the resulting solution of chitosan/squalene/surfactant/antigen was mixed by vortexing or syringe aspiration.

Example 6

Comparative Immunopotentiation

The following experiments were conducted to assess the immune response to an antigen that has been incorporated in the adjuvant disclosed in Example 5 versus the adjuvant disclosed in Example 3.

Female BALB/c mice (n=10) received intraperitoneal injections of 25 μg of histidine-tagged recombinant βeta subunit of human chorionic gonadotropin (rβhCG) expressed in yeast, administered in either the adjuvant disclosed in Example 5 or the adjuvant disclosed in Example 3. Primary immunization occurred on day 0 with a second injection on day 21.

Antibody titers were determined in an antibody capture enzyme-linked immunosorbent assay (ELISA) using either rβhCG (1 μg/ml) or native human chorionic gonadotropin (hCG:Sigma Chemicals, St Louis, MO). ELISA results, which are shown in Table 5, indicate that the presently claimed adjuvant using phosphate buffer (Example 5) is equivalent to the adjuvant using sodium hydroxide (set forth in Example 3) with respect to potentiating an immune response (no statistical difference between GRP I-r and GRP II-r; no statistical difference between GRP I-n and GRP II-n). GRP I-r refers to the response to rβhCG elicited by the administration of rβhCG using the adjuvant with phosphate buffer, while GRP II-r refers to the response to rβhCG elicited by administration of rβhCG using the adjuvant containing sodium hydroxide. GRP I-n refers to the response to native human chorionic gonadotropin elicited by the administration of rβhCG using the adjuvant with phosphate buffer, while GRP II-n to the response to native human chorionic gonadotropin elicited by the administration of rβhCG using the adjuvant containing sodium hydroxide.

Although the present invention has been described in terms of preferred embodiments, it is intended that the present invention encompass all modifications and variations which occur to those skilled in the art upon consideration of the disclosure herein, and in particular those embodiments which are within the broadest proper interpretation of the claims and their requirements.

Table 5 Comparative Immunopotentiation

(data express as geometric mean)

	Day 21	Day 42	Day 55	Day 69	Day 89	Day 110	Day 138	Day 188
GRP I-r	5788	97616	51693	40304	46296	30544	15797	51693
GRP II-r	2425	106362	62343	34045	75703	32000	22601	53017
GRP I-n	137	42	2757	2013	4897	4264	4624	4263
GRP II-n	174	47	4624	763	2894	7276	2268	6101

WHAT IS CLAIMED IS:

1. A method of potentiating an immune response in an animal, said method comprising the steps of:

a) preparing a chitosan solution;

b) preparing an alkaline solution;

c) preparing an oil/surfactant solution, wherein the oil can be metabolically degraded;

d) mixing the chitosan solution with the alkaline solution, the oil/surfactant solution, and an antigen to form an emulsion; and

e) administering the emulsion to an animal by a route of administration that permits the animal to mount an immune response to the antigen.

2. The method of claim 1, wherein the oil is squalene.

3. The method of claim 1, wherein the pH of the chitosan solution is about 5.0.

4. The method of claim 1 wherein the route of administration is selected from the groups consisting of intramuscular injection, intraperitoneal injection and subcutaneous injection.

5. The method of claim 1, wherein the animal is human.

6. The method of claim 1, wherein the alkaline solution is selected from the group consisting of phosphate buffer and HEPES buffer.

7. A composition for potentiating an immune response, said composition comprising antigen, an alkaline solution, oil, surfactant, and chitosan solution, wherein said oil can be metabolically degraded.

8. The method of claim 7, wherein the oil is squalene.

9. The composition of claim 7, wherein the pH of the chitosan solution is about 5.0.

10. The composition of claim 7, where the alkaline solution is selected from the group consisting of phosphate buffer and HEPES.

11. An immunogen produced by the process of:

a) preparing a chitosan solution;

b) preparing an alkaline solution;

c) preparing an oil/surfactant solution, wherein the oil can be metabolically degraded; and

d) mixing the chitosan solution with the alkaline solution, the oil/surfactant solution, and an antigen to form an emulsion.

12. The immunogen of claim 11, wherein the oil is squalene.

13. The immunogen of claim 11, wherein the pH of the chitosan solution is about 5.0.

14. The immunogen of claim 11, wherein the alkaline solution is selected from the group consisting of phosphate buffer and HEPES.

15. A method for producing an immunogen comprising the step:

a) preparing a chitosan solution;

b) preparing an alkaline solution;

c) preparing an oil/surfactant solution, wherein the oil can be metabolically degraded; and

d) mixing the chitosan solution with the alkaline solution, the oil/surfactant solution, and an antigen to form an emulsion.

16. The method of claim 15, wherein the oil is squalene.

17. The method of claim 15, wherein the pH of the chitosan solution is about 5.0.

18. The method of claim 15, wherein the alkaline solution is selected from the group consisting of phosphate buffer and HEPES.

19. A kit comprising

a) a chitosan solution;

b) an alkaline solution; and

c) an oil/surfactant solution, wherein the oil can be metabolically degraded.

20. The kit of claim 19, wherein the oil is squalene.

21. The kit of claim 19, wherein the pH of the chitosan solution is about 5.0.

22. The kit of claim 19, wherein the alkaline solution is selected from the group consisting of phosphate buffer and HEPES.

23. An adjuvant prepared by the process of mixing a chitosan solution with an alkaline solution and an oil/surfactant solution to form an emulsion, wherein the oil can be metabolically degraded.

24. The adjuvant of claim 23, wherein the oil is squalene.

25. The adjuvant of claim 23, wherein the pH of the chitosan solution is about 5.0.

26. The adjuvant of claim 23, wherein the alkaline solution is selected from the group consisting of phosphate buffer and HEPES.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANTS: ZONAGEN, INC.

(ii) TITLE OF INVENTION: Chitosan Induced Immunopotentiation

(iii) NUMBER OF SEQUENCES: 6

(iv) CORRESPONDENCE ADDRESS:
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(C) CITY: Chicago
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(E) COUNTRY: United States of America
(F) ZIP: 60606-6402

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(viii) ATTORNEY/AGENT INFORMATION:
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(B) TELEFAX: 312-474-0048

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: not relevant
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Gly Gln His Lys Pro Glu Ala Pro Asp Tyr Ser Ser
1 5 10

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: not relevant
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Arg Gly Asp Cys Glu Gly Leu Gly Cys Cys Tyr Ser
1 5 10

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Ala Pro Asp Thr Asp Trp Cys Asp Ser Ile Pro Ala Arg Asp Arg Leu
1 5 10 15

Pro Cys

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Asp Arg Ala Val Tyr Glu Asn Glu Leu Val Ala Thr
1 5 10

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Met Pro Val Gly Val Glu Gly Ala Gly Ala Ala Glu
1 5 10

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Val Ser Ser Lys Gly Pro Met Ile Leu Leu Gln Ala Thr Lys Asp Pro
1 5 10 15

Pro Glu Lys Leu Arg Val Pro Val
20

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(54) Title: CHITOSAN INDUCED IMMUNOPOTENTIATION

(57) Abstract: Methods and compositions for potentiating an immune response are disclosed which incorporate chitosan as an immunopotentiating adjuvant. Administration of the compositions of the invention is effected by various routes.

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B. FIELDS SEARCHED

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Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 98 42374 A (ZONAGEN INC) 1 October 1998 (1998-10-01) the whole document ---	1-26
X	US 5 965 144 A (PODOLSKI JOSEPH S ET AL) 12 October 1999 (1999-10-12) examples 3,4 ---	1-26
P,X	SEFERIAN P G ET AL: "Immune stimulating activity of two new chitosan containing adjuvant formulations" VACCINE, vol. 19, no. 6, 8 November 2000 (2000-11-08), pages 661-668, XP004219905 the whole document --- -/--	1-26

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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'A' document defining the general state of the art which is not considered to be of particular relevance

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European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Teyssier, B

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 00/41132

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	WO 99 65521 A (ZONAGEN INC) 23 December 1999 (1999-12-23) claims 87-107; example 6	1-26

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Information on patent family members

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Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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		WO 9965521 A1	23-12-1999